

A Novel Human Fibroblast Growth Factor Treats Experimental Intestinal Inflammation

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Background & Aims: We recently identified a novel member of the human fibroblast growth factor (FGF) family of signaling molecules, designated FGF-20. In the present study, we examined the activity of this protein in 2 animal models of acute intestinal inflammation and in mechanistic studies in vitro. **Methods:** In vivo experiments consisted of a murine dextran sulfate sodium (DSS) model of colitis and a rat indomethacin model of small intestinal ulceration/inflammation. Cell growth, restitution, gene expression (cyclooxygenase-2 [COX-2] and intestinal trefoil factor [ITF]), and prostaglandin E₂ (PGE₂) levels were examined in vitro. **Results:** In the DSS-colitis model, prophylactic administration of FGF-20 significantly reduced the severity and extent of mucosal damage as indicated by a 55%–93% reduction in luminal blood loss, distal colonic edema, histologic inflammation, and epithelial cell loss relative to animals administered vehicle control. No toxicity was noted during administration of FGF-20 to normal controls. In addition, therapeutic administration of FGF-20 enhanced survival in this model. In the indomethacin–small bowel ulceration/inflammation model, administration of FGF-20 reduced small intestinal weight gain, necrosis, inflammation, and weight loss (36%–53% relative to vehicle control). In vitro studies demonstrated that FGF-20 stimulates growth, restitution, mRNA expression of COX-2 and ITF, and PGE₂ levels in human intestinal epithelial cells and enhances the growth of human intestinal fibroblasts. **Conclusions:** FGF-20, having demonstrated therapeutic activity in 2 experimental models of intestinal inflammation, represents a promising new candidate for the treatment of human inflammatory bowel disease.

Inflammatory bowel disease (IBD) comprises a spectrum of immune-mediated chronic gastrointestinal disorders, including ulcerative colitis and Crohn's disease.^{1–3} In IBD, the integrity of the intestinal mucosa is compromised, and healing of the surface epithelium is accomplished via epithelial cell migration ("restitution"),

proliferation, and differentiation.⁴ Stimulated fibroblasts are believed to play a role in the healing process.⁴

Although many treatments for IBD exist, additional therapeutic approaches are needed because many patients either do not respond to current options or develop significant side effects to medications, thereby precluding their continued use. Because of the inadequacy of current therapies, some IBD patients with refractory disease undergo surgery to remove a portion of the intestine. A new agent that has fewer side effects than current approaches, has sustainable efficacy in patients unresponsive to available drugs, targets therapeutic mechanisms distinct from current medications, and eliminates the need for surgery would offer clinical and pharmacoeconomic benefits. Because the integrity of the intestinal mucosa is breached in IBD, thereby potentiating the uptake of injurious luminal bacterial antigens and cell wall polymers,¹ it follows that agents that facilitate or accelerate epithelial repair may be therapeutically useful. No currently marketed products fall into this category.

One relevant class of agents for the repair of intestinal epithelium is that of the peptide growth factors, including, among others, members of the fibroblast growth factor (FGF) family.^{5,6} FGF receptors are present on intestinal epithelium,^{7,8} and enhanced expression of various FGF family members has been demonstrated in the intestines of IBD patients,^{9–12} suggesting a potential endogenous reparative role for this family of growth factors after injury caused by inflammatory processes.

Abbreviations used in this paper: COX-2, cyclooxygenase-2; DMEM, Dulbecco's modified Eagle medium; DSS, dextran sulfate sodium; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; FGF, fibroblast growth factor; IBD, inflammatory bowel disease; ITF, intestinal trefoil factor; PBS, phosphate-buffered saline; PGE₂, prostaglandin E₂; RT-PCR, reverse-transcription polymerase chain reaction.

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Support for this theory comes from in vitro and in vivo studies demonstrating that FGFs enhance the restitution and proliferation of intestinal epithelium.^{7,13-15} Assuming that naturally occurring reparative factors such as FGFs are present in suboptimal quantities in the intestines of IBD patients, it follows that supplementation with these factors may promote healing and thus alleviate the symptoms associated with IBD. FGFs have in fact shown promise in animal models of IBD.^{13,16-18}

We recently identified and characterized a novel member of the human FGF family that we designate FGF-20.¹⁹ This factor interacts with multiple FGF receptors and displays mitogenic activity on fibroblasts and epithelial cells. In the present study, we examined the in vivo effects of FGF-20 in 2 rodent models of IBD: dextran sulfate sodium (DSS) treatment of mice to induce an ulcerative colitis-like syndrome and indomethacin treatment of rats to induce ulceration and inflammation of the small bowel, as is seen in Crohn's disease. We also performed in vitro studies to explore the mechanisms of action of FGF-20.

Materials and Methods

Purification of FGF-20

The human FGF-20 cDNA¹⁹ was cloned into pETMY, a modified pRSET vector (Invitrogen, San Diego, CA). The resulting construct encodes amino acids 2-211 of FGF-20 preceded by the sequence MRGSHHHHHHGMASMTGGQQMGRDLYDDDDDKDRWGS, which contains a histidine tag used for purification purposes, as well as additional vector-encoded residues. The vector was transformed into *Escherichia coli* strain BL21 (Novagen, Madison, WI), which was grown to an optical density of 0.6 and infected with CE6 bacteriophage lambda (Novagen) at a multiplicity of infection of 5. The infected bacterial culture was further incubated for 3 hours at 27°C, obtained by centrifugation (4000×g for 15 minutes at 4°C), resuspended in phosphate-buffered saline (PBS) + 0.5 mol/L NaCl + 1.0 mol/L L-arginine, and disrupted with 2 passes through a microfluidizer at 8000 pounds per square inch. Cell debris was removed by centrifugation (10,000 × g for 25 minutes at 4°C) and discarded. The resulting supernatant containing the FGF-20 protein was clarified by filtration through a 0.22-μm low protein-binding filter and loaded onto a column containing nickel-charged Sepharose (Pharmacia Biotech, Piscataway, NJ). The column was washed with PBS + 0.5 mol/L NaCl + 1.0 mol/L L-arginine, and bound protein was eluted with a linear gradient of 0-0.5 mol/L imidazole. Fractions containing FGF-20 were pooled, dialyzed at 4°C against PBS + 1.0 mol/L L-arginine, and loaded onto a column containing uncharged Sepharose (Pharmacia Biotech). FGF-20 was captured in the flowthrough, dialyzed at 4°C against PBS + 1.0 mol/L L-arginine, and sterilized by passage through a 0.22-μm low protein-binding filter. Purified FGF-20 had an

endotoxin level of ≤25 endotoxin units/mg as determined by the Limulus amoebocyte lysate assay (BioWhittaker, Walkersville, MD).

Bromo-Deoxy-Uridine Assay

NIH 3T3 murine embryonic fibroblasts and Balb/MK murine keratinocytes were cultured in 96-well plates to approximately 100% confluence in growth media (NIH 3T3 cells: Dulbecco's modified Eagle medium [DMEM] + 10% bovine calf serum [Invitrogen]; Balb/MK cells: keratinocyte-SFM [Invitrogen]). Before adding FGF-20, Balb/MK cells were prestarved for 24 hours in basal keratinocyte media. FGF-20 was added to cells for 18 hours in the appropriate basal media supplemented with 0.1% bovine serum albumin, and the bromo-deoxy-uridine assay was performed according to the manufacturer's specifications (Roche Molecular Biochemicals, Indianapolis, IN) using a 3 hour bromo-deoxy-uridine incorporation time.

Animals

Mice. Six- to 8-week-old female Balb/c mice weighing 20-22 g were obtained from Harlan Labs (Indianapolis, IN) for use in the DSS model. Between 3 and 5 animals were housed per cage in polycarbonate cages with filter tops and given mouse chow (Harlan Teklab, Madison, WI) and tap water ad libitum. Animals were acclimated for 6 days before experimental use and were sacrificed by CO₂ inhalation at the end of the study.

Rats. Female Lewis rats weighing 175-200 g were obtained from Harlan Labs for use in the indomethacin model. Four animals were housed per cage and given Harlan Teklab rat chow and tap water ad libitum. Animals were acclimated for 8 days before experimental use. At the end of the study, animals were anesthetized with isoflurane and sacrificed by cervical dislocation after blood collection.

Murine DSS Model

DSS (Spectrum Chemicals, Gardena, CA) working solutions were freshly made every other day in tap water and stored at 4°C. FGF-20 was diluted in PBS + 1.0 mol/L L-arginine, and the vehicle solution consisted of PBS + 1.0 mol/L L-arginine. Intraperitoneal (IP) and subcutaneous (SC) injections were both performed in volumes of 10 mL/kg using FGF-20 stocks of the appropriate concentrations so as to achieve the desired final concentration of FGF-20 (5, 1, or 0.2 mg/kg, as indicated in the Results section and in the Figures). At necropsy, the colon was removed, and colon blood content was scored according to the following criteria: 0, normal to semisolid stool, no blood; 1, normal to semisolid stool, blood-tinged; 2, semisolid to fluid stool with definite evidence of blood; 3, bloody fluid. For histopathologic examination, 3 distal colonic regions spaced approximately 1 cm apart were collected into 10% neutral buffered formalin, processed for paraffin embedding, sectioned, and stained with hematoxylin and eosin. Each section was scored for various parameters, and the mean of the scores for each of the regions was determined.

Submucosal edema was quantitated by measuring the distance from the muscularis mucosa to the internal border of the outer muscle layer. Inflammation (foamy macrophage, lymphocyte, and polymorphonuclear cell infiltrate) was assigned a severity score according to the following criteria: 0, normal; 1, minimal; 2, mild; 3, moderate; 4, marked; 5, severe. Glandular epithelial loss and surface epithelial loss were scored using the following criteria: 0, normal; 1, 1%–10% of the mucosa affected; 2, 11%–25% of the mucosa affected; 3, 26%–50% of the mucosa affected; 4, 51%–75% of the mucosa affected; 5, 76%–100% of the mucosa affected. The 3 important scored parameters (inflammation, glandular epithelial loss, and surface epithelial loss) were combined to arrive at an overall histopathology score that indicates the overall damage and could have a maximum score of 15. For each animal, 3 distal colonic regions spaced approximately 1 cm apart were scored, and the means of the scores for each of the regions were determined.

Rat Indomethacin Model

To induce disease, indomethacin (Sigma, St. Louis, MO) was prepared in 5% sodium bicarbonate to 7.5 mg/mL and injected SC into rats on 2 consecutive days in a volume of 1 mL/kg so as to achieve the desired final concentration of 7.5 mg/kg/dose.¹³ FGF-20 was diluted in PBS + 1.0 mol/L L-arginine, and the vehicle solution consisted of PBS + 1.0 mol/L L-arginine + 5 mg/mL bovine serum albumin. Intravenous (IV) tail vein injections were performed in a volume of 1 mL/kg using FGF-20 stocks of the appropriate concentrations so as to achieve the desired final concentration of FGF-20 (5, 1, or 0.2 mg/kg as indicated in the Results section and in the Figures). At necropsy, a 10-cm section of the distal jejunum in the area at risk for lesions was removed and weighed. This jejunum fragment was then used to obtain 5 approximately equally spaced sections that were collected into 10% neutral buffered formalin, processed for paraffin embedding, sectioned, and stained with hematoxylin and eosin for histopathologic examination. Necrosis was scored according to the following criteria: 0, normal; 1, 1%–10% mucosal necrosis; 2, 11%–25% mucosal necrosis; 3, 26%–50% mucosal necrosis; 4, 51%–75% mucosal necrosis; 5, 76%–100% mucosal necrosis. Inflammation was scored according to the following criteria: 0, none; 1, minimal inflammation in mesentery and muscle or lesion; 2, mild inflammation in mesentery and muscle or lesion; 3, moderate inflammation in mesentery and muscle or lesion; 4, marked inflammation in the lesion; 5, severe inflammation in the lesion.

Growth Assay

CCD-18Co normal human colonic fibroblasts and FHs 74 Int normal human small intestinal epithelial cells were plated in 6-well plates to approximately 25% confluence in growth media and allowed to attach overnight (CCD-18Co cells: DMEM + 10% FBS [Invitrogen]; FHs 74 Int cells: DMEM + 10% FBS + nonessential amino acids [Invitrogen] + sodium pyruvate [Invitrogen] + 1 mmol oxalacetic acid

[Sigma] + 0.2 U/mL insulin [Invitrogen]). The next day, the growth media was removed and replaced with a 1:1 mixture of DMEM (without or with FGF-20)/growth media. The cells were fed with fresh factor after 3 days and counted after 6 days.

Wounded Monolayer Repair Assay

An in vitro healing assay was performed using a modified version of a published method.^{13,20} Briefly, reference lines were drawn horizontally across the outer bottom of 24-well plates. HT-29 and Caco-2 human colon carcinoma cells were plated and grown to confluence in DMEM + 5% FBS, and then incubated for 24 hours in DMEM + 0.1% FBS. Linear "wounds" were made with a sterile plastic pipette tip perpendicular to the lines on the bottom of the wells. Then 10% FBS (positive control) or FGF-20 was added, and the size of the wound was measured microscopically at various times at predetermined locations corresponding to the reference lines.

Determination of Cyclooxygenase-2 and Intestinal Trefoil Factor Gene Expression by Reverse-Transcription Polymerase Chain Reaction

Cells (HT-29 and Caco-2) were plated and grown to confluence in DMEM + 5% FBS, and then incubated for 24 hours in DMEM + 0.1% FBS. FGF-20 (100 ng/mL) was then added, and total RNA was obtained from the cells after various times using Trizol (Invitrogen) according to the manufacturer's instructions. RNA was reverse-transcribed using 2 µg of total RNA, 15 U of RNA inhibitor, first-strand synthesis buffer (Invitrogen), 5 mmol deoxynucleoside triphosphate (Pharmacia, Upsala, Sweden), 125 pmol random hexamer primers (Pharmacia), and 125 U of Moloney murine leukemia virus reverse transcriptase (Invitrogen) in a final volume of 25 µL. The reaction was performed for 1 hour at 39°C, followed by 7 minutes at 93°C and 1 minute at 24°C, and then slowly cooled to 4°C for 20 minutes. Reverse transcription-polymerase chain reaction (RT-PCR) was performed in a volume of 50 µL containing 5 µL of reverse transcriptase mixture, 1× Taq buffer, 5 pmol of each primer, 2.5 mmol deoxynucleoside triphosphate, and 1 unit of Taq polymerase. The primers used to amplify human cyclooxygenase-2 (COX-2), intestinal trefoil factor (ITF), and β-actin were as follows:

COX-2: sense, 5'-AGATCATCTCTGCCTGAGTATCTT-3'; antisense, 5'-TTCAAATGAGATTGTGGGAAAATTGCT-3'

ITF: sense, 5'-GTGCCAGCCAAGGACAG-3'; antisense, 5'-CGTTAAGACATCAGCCTCCAG-3'

β-actin: sense, 5'-CCAACCGCAAGAAGATGA-3'; antisense, 5'-GATCTTCATGAGGTAGTCAGT-3'.

RT-PCR was carried out in a Perkin-Elmer 9600 cyclor (Perkin-Elmer, Wellesley, MA) programmed for 20–40 cycles to assess the linearity of the amplification. The PCR products were separated on 2% Tris-acetate/EDTA agarose gels containing gel star fluorescent dye (FMC, Philadelphia, PA). A negative from the gels was taken with an Alphamager 2000 (Alpha Innotech, San Leandro, CA).

Determination of Prostaglandin E₂ Levels by Enzyme-Linked Immunosorbent Assay

Cells (HT-29 and Caco-2) were plated and grown to confluence in DMEM + 5% FBS, and then incubated for 24 hours in DMEM + 0.1% FBS. FGF-20 was then added, and 24 hours later the culture medium was harvested and assessed for prostaglandin E₂ (PGE₂) levels via enzyme-linked immunosorbent assay (ELISA; Assay Designs, Ann Arbor, MI).

Results

Expression, Purification, and In Vitro Activity of Recombinant FGF-20

Purified human FGF-20 was isolated from *Escherichia coli* engineered to express full-length FGF-20 protein (Figure 1A). The recombinant protein, which contains vector-encoded sequences and a histidine tag at the N-terminus, had a molecular weight of approximately 29 kilodaltons (Figure 1A, Lane 2), close to its predicted molecular weight of 27,739 daltons. Purified FGF-20 was biologically active, as demonstrated by its ability to induce DNA synthesis in murine fibroblasts (NIH 3T3) and epithelial cells (Balb/MK) at half maximal concentrations of approximately 5 ng/mL (Figure 1B). Similar biological activity was obtained with purified recombinant full-length FGF-20 devoid of vector-encoded sequences (data not shown).

Prophylactic Administration of FGF-20 Is Active in a DSS-Mediated Murine Model of Colitis

The effect of FGF-20 on colitis was initially examined in a murine DSS-mediated disease model.^{21,22} In this model, Balb/c mice exposed to DSS for 7 days developed distal colonic inflammation and edema in association with crypt and colonic glandular epithelial loss, erosion, and ulceration, leading to hemorrhage. In this study, DSS-associated effects on the proximal colon were much less severe than on the distal colon and thus are not reported. FGF-20 (5 mg/kg) administered daily via IP injections on each of the 7 days of DSS exposure significantly reduced the extent and severity of mucosal damage (Figure 2). Specifically, FGF-20 resulted in the following protective effects on the distal colon: 93% reduction of blood content scores, reflecting hemorrhagic diarrhea; 76% reduction in submucosal edema; 55% reduction in mucosal inflammation; 57% reduction in glandular epithelial loss; and 84% reduction in surface epithelial loss. FGF-20 administration also inhibited the DSS-induced decrease in colon length. Histopathology sum scores that take into consideration the parameters of inflammation, glandular epithelial loss, and erosion in-

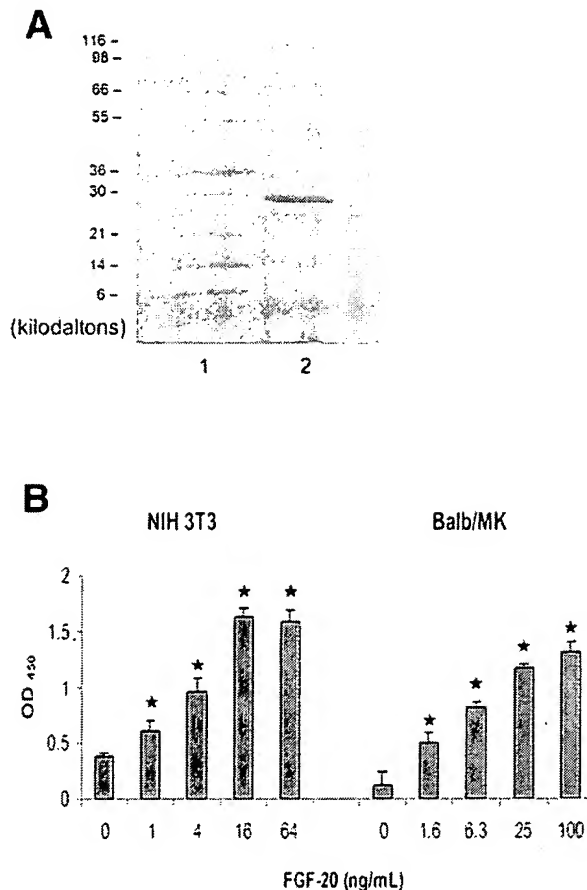


Figure 1. Purification and in vitro biological activity of FGF-20. (A) Full-length FGF-20 protein possessing an N-terminal histidine tag was expressed in *Escherichia coli* and purified to near homogeneity by nickel chromatography. Five micrograms of FGF-20 was resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (4%–20% gel) under reducing conditions and visualized with Coomassie stain (lane 2). Lane 1 depicts protein standards (in kilodaltons). (B) NIH 3T3 murine fibroblasts and Balb/MK murine keratinocytes were incubated with purified FGF-20 at the indicated concentrations for 18 hours and analyzed by a bromo-deoxy-uridine incorporation assay. Data points represent the mean of triplicate wells \pm standard deviation. Changes that are statistically different from control cells receiving no factor ($P < 0.05$ by the 2-tailed Student *t* test) are indicated with a star.

dicate that FGF-20 caused a 66% reduction in DSS-mediated effects on the distal colon. Finally, FGF-20 administration reduced the amount of DSS-induced weight loss by 30%. A representative histopathologic example of the protective effect of FGF-20 on the distal colon is depicted in Figure 3, which shows that FGF-20 inhibited the mucosal changes and submucosal edema associated with DSS treatment.

In a follow-up murine DSS-colitis study, we sought to verify the initial results and to determine optimal dosing in the prophylactic protocol using a SC delivery method. Mice were exposed to DSS for 7 days, and FGF-20 (5, 1,

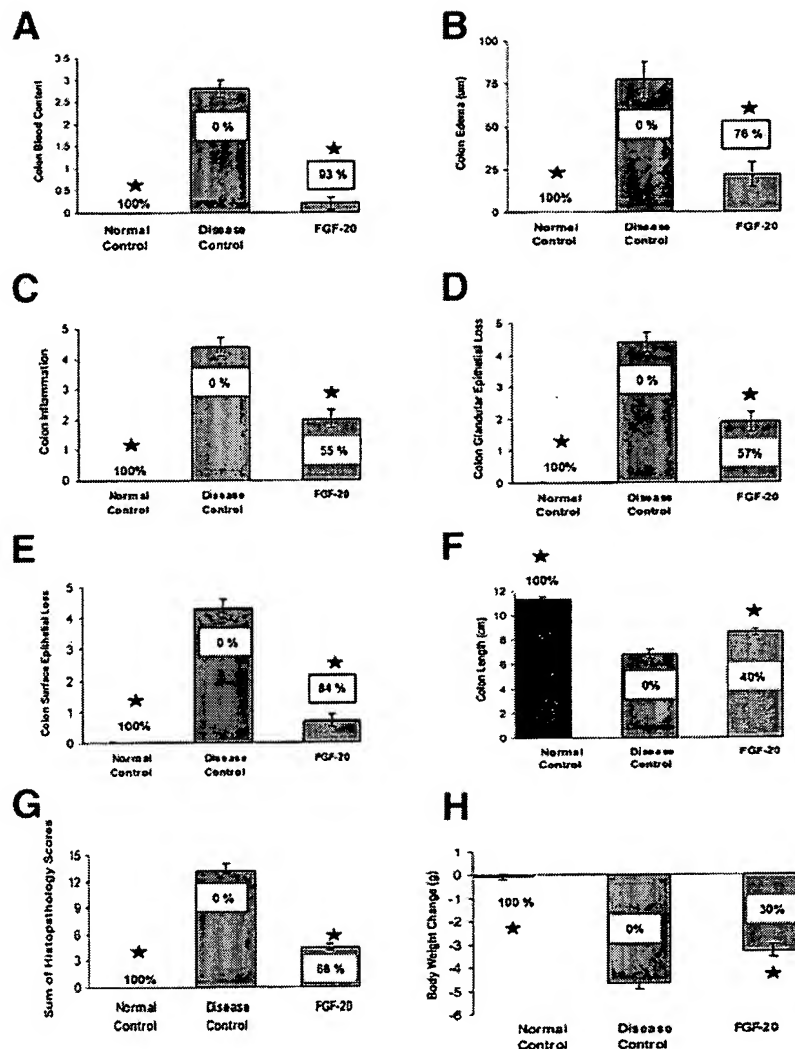


Figure 2. Effects of FGF-20 on DSS-induced colitis. To induce colitis, female Balb/c mice were exposed to 5% DSS in drinking water for 7 days (day 0 to 6). Disease control animals ($n = 10$) received daily IP injections of vehicle solution on each day of DSS exposure. The FGF-20 group of animals ($n = 10$) received daily IP injections of FGF-20 (5 mg/kg) on each day of DSS exposure. Normal control animals ($n = 5$) were not exposed to DSS, but did receive daily IP injections of vehicle solution on day 0 to 6. Animals were sacrificed on day 7, and the distal colon was scored for the following parameters (see Materials and Methods for details): (A) blood content, (B) submucosal edema, (C) mucosal inflammation, (D) glandular epithelial loss, (E) surface epithelial loss (erosion), and (F) length. (G) An overall histopathology score that takes into consideration inflammation, glandular epithelial loss, and erosion in the distal colon. (H) The change in total body weight from day 0 to 7. Results are reported as mean \pm standard error, and the percentage changes indicated are relative to the disease control group. Changes that are statistically different from the disease control group ($P < 0.05$ by the 2-tailed Student t test) are indicated with a star.

and 0.2 mg/kg) was administered daily via SC injections on each of the 7 days of DSS exposure (Figure 4). As was seen in the initial study, FGF-20 treatment reduced the extent and severity of mucosal damage as measured by fecal blood, histologic injury, and colon length, and did so in a dose-dependent fashion with maximum protection offered at the highest FGF-20 concentration examined (5 mg/kg). No significant protective effect of FGF-20 on DSS-induced weight loss was observed in this follow-up study. Significant protection from mucosal

damage was also seen with FGF-20 at 1 mg/kg, whereas 0.2 mg/kg of this factor provided little protection from DSS-induced colitis.

Similar protective effects were obtained with purified recombinant full-length FGF-20 devoid of vector-encoded sequences. Moreover, following the administration of this FGF-20 protein (5 mg/kg SC once daily for 7 days) to normal nondisease control animals, an analysis of animal weight, blood hematology/clinical chemistry, and histopathology on 28 different tissues

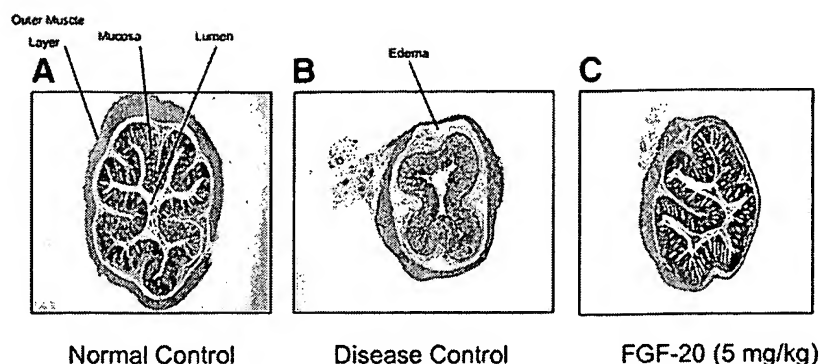


Figure 3. Effects of FGF-20 on DSS-induced colitis: histopathology. Representative sections of distal colon were collected at necropsy, preserved in formalin, stained with H&E, magnified 50 \times , and photographed. The groups are as described in the legend to Figure 2. Note that FGF-20 (5 mg/kg) inhibits the mucosal changes and submucosal edema associated with DSS treatment.

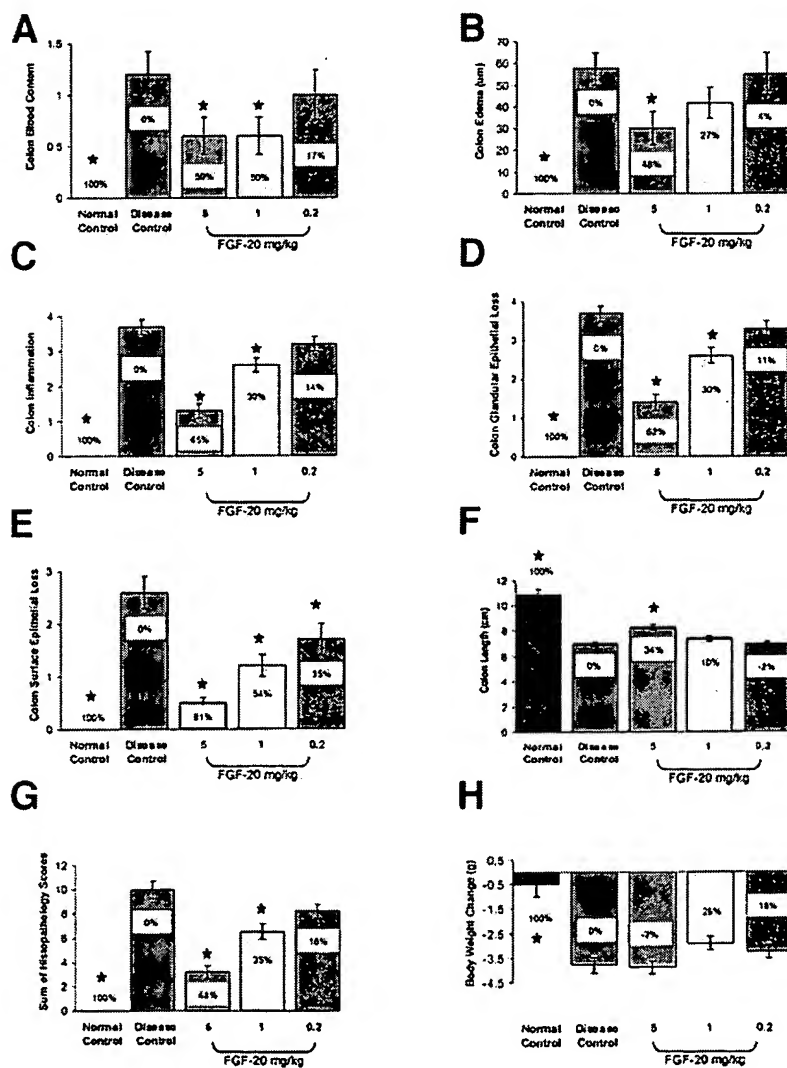


Figure 4. Effects of FGF-20 on DSS-induced colitis: dose response. To induce colitis, female Balb/c mice were exposed to 4% DSS in drinking water for 7 days (day 0 to day 6). Disease control animals ($n = 9$) received daily SC injections of vehicle solution on each day of DSS exposure. The FGF-20 group of animals ($n = 9$) received daily SC injections of FGF-20 at the indicated concentrations on each day of DSS exposure. Normal control animals ($n = 4$) were not exposed to DSS, but did receive daily SC injections of vehicle solution on day 0 to day 6. Animals were sacrificed on day 7, and the distal colon was scored for the following parameters (see Materials and Methods for details): (A) blood content, (B) submucosal edema, (C) mucosal inflammation, (D) glandular epithelial loss, (E) surface epithelial loss (erosion), and (F) length. (G) An overall histopathology score that takes into consideration inflammation, glandular epithelial loss, and erosion in the distal colon. (H) The change in total body weight from day 0 to 7. Results are reported as mean \pm standard error, and the percentage changes indicated are relative to the disease control group. Changes that are statistically different from the disease control group ($P < 0.05$ by the 2-tailed Student t test) are indicated with a star.

revealed that the only gross tissue alteration induced by FGF-20 was increased injection site inflammation/fibroplasia. This analysis also indicated that FGF-20 induced a moderate increase in absolute neutrophils and cholesterol. (Mean absolute neutrophil counts in FGF-20-treated, vehicle control and nontreated control animals were 1405, 968, and 1130, respectively; *t* test *P* value = 0.030 for comparison of FGF-20-treated to vehicle control. Mean cholesterol counts in FGF-20-treated, vehicle control and nontreated control animals were 100, 79.2, and 77.2, respectively; *t* test *P* value = 0.024 for comparison of FGF-20-treated to vehicle control). The biological relevance of these findings remains to be determined.

Therapeutic Administration of FGF-20 Enhances Survival in the Murine DSS Model

In the experiments described previously, DSS exposure and FGF-20 administration were initiated simultaneously on day 0. In another experiment, the effect of FGF-20 administered after the initiation of DSS treatment was examined. To this end, Balb/c mice exposed to DSS for 7 days (day 0 to day 6) were injected daily SC with various concentrations of FGF-20 (5, 1, and 0.2 mg/kg) beginning on the fifth day of DSS exposure (i.e., day 4) and ending 3 days after the termination of DSS exposure (i.e., day 9). Animal survival was recorded on a daily basis, and the experiment was concluded on day 10. As shown in Figure 5, therapeutic administration of FGF-20 at 5 mg/kg enhanced survival relative to the disease control group. Thus, whereas only 44% (4 of 9) of the animals in the disease control group survived until the end of the study, 89% (8 of 9) of the animals treated with FGF-20 at 5 mg/kg survived. FGF-20 administered therapeutically at lower doses (1 and 0.2 mg/kg) had little or no effect on survival.

FGF-20 Is Active in an Indomethacin-Mediated Rat Model of Small Bowel Ulceration/Inflammation

Treatment of susceptible Lewis rats with indomethacin results in chronic small intestinal linear ulcerations bearing some similarity to those observed in Crohn's disease. This model was used to examine the ability of FGF-20 to treat discrete mucosal ulcers. To this end, Lewis rats treated with indomethacin (7.5 mg/kg SQ) for 2 days (day 0 to day 1) were injected daily IV with various concentrations of FGF-20 (5, 1, 0.2 mg/kg) beginning on the day before the initiation of indomethacin treatment (i.e., day -1) and ending 3 days after the termination of indomethacin treatment (i.e.,

day 4). Animals were sacrificed and examined on day 5. Administration of FGF-20 at 0.2 mg/kg resulted in the following protective effects relative to vehicle-treated disease control animals: 52% reduction in indomethacin-induced small intestine weight increase (as measured from a 10-cm section of distal jejunum taken from the area at risk), 53% reduction in histopathologic intestinal necrosis, and a 38% reduction in histopathologic intestinal inflammation (Figure 6). This concentration of FGF-20 also significantly reduced the indomethacin-induced increase in blood neutrophils by 39% and inhibited weight loss by 36%. Higher concentrations of FGF-20 (i.e., 1 and 5 mg/kg) were less active in this model than the 0.2 mg/kg dose. A representative histopathologic example of the protective effect of FGF-20 on the small intestine is depicted in Figure 7, which shows that FGF-20 inhibits the mucosal ulceration and necrosis normally associated with indomethacin treatment.

FGF-20 Enhances the Growth and Restitution of Intestinal Cells In Vitro

Because the healing of the surface mucosa involves epithelial restitution, as well as epithelial and fibroblast growth and/or activation, we examined the effect of FGF-20 on these processes in vitro. Our findings demonstrate that FGF-20 significantly enhances the growth of normal human colonic fibroblasts (CCD-18Co) and normal human intestinal epithelial cells (FHs Int 74) (Figure 8). This effect of FGF-20 was dose-dependent and resulted in a 2- to 3-fold increase in cell number over the course of 6 days of culture.

To explore the effects of FGF-20 on restitution, a wounded monolayer repair assay was performed on 2 human colonic epithelial cancer cell lines, HT-29 and Caco-2. The results of this assay demonstrate that FGF-20 significantly stimulates wound closure in a concentration-dependent manner (Figure 9). The highest FGF-20 dose examined (100 ng/mL) stimulated closure to a similar degree as that of the positive control (10% FBS). Consistent with these results, FGF-20 was also found to enhance the migration of FHs Int 74 normal human intestinal epithelial cells in a dose-dependent fashion when examined in a modified Boyden chamber assay (data not shown).

FGF-20 Stimulates COX-2 and ITF Expression and PGE₂ Levels in Colonic Cells In Vitro

We next examined the effect of FGF-20 on the mRNA expression of COX-2 and ITF, 2 genes whose

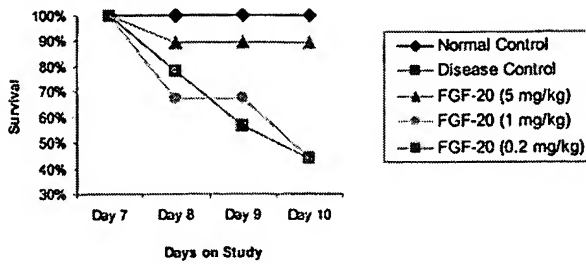


Figure 5. Effect of therapeutically administered FGF-20 on survival in the DSS model of colitis. Female Balb/c mice were exposed to 4% DSS in drinking water for 7 days (day 0 to 6) and then switched to normal drinking water for 4 additional days (day 7 to 10). Disease control animals ($n = 9$) received daily SC injections of vehicle solution on day 4 to day 9. FGF-20 groups ($n = 9$) received daily SC injections of the indicated concentrations of FGF-20 on day 4 to 9. Normal control animals ($n = 3$) were not exposed to DSS, but did receive daily SC injections of vehicle solution on day 4 to 9. Animal survival was recorded daily, and the experiment was concluded on day 10. Note that the disease control and the 0.2 mg/kg FGF-20 groups yielded identical results and are both represented by red squares.

protein products exert a protective effect in intestinal inflammation.^{23,24} The result of this experiment indicates that FGF-20 (100 ng/mL) stimulates the expression of both of these genes in HT-29 and Caco-2 human colonic epithelial cancer cells (Figure 10). Peak up-regulation was seen following exposure of cells to FGF-20 for 1–3 hours (COX-2) or 3–6 hours (ITF). An increase in COX-2 protein expression after exposure of HT-29 and Caco-2 cells to FGF-20 for 3 hours was evident via Western blot analysis (data not shown).

Because prostaglandins have been implicated in mucosal healing²³ and PGE₂ production is stimulated by COX-2, we examined the effect of FGF-20 on PGE₂ levels in HT-29 and Caco-2 cells. The result of this experiment indicates that FGF-20 significantly enhances the levels of PGE₂ in a dose-dependent fashion in both of these cell lines. The highest FGF-20 dose examined (100

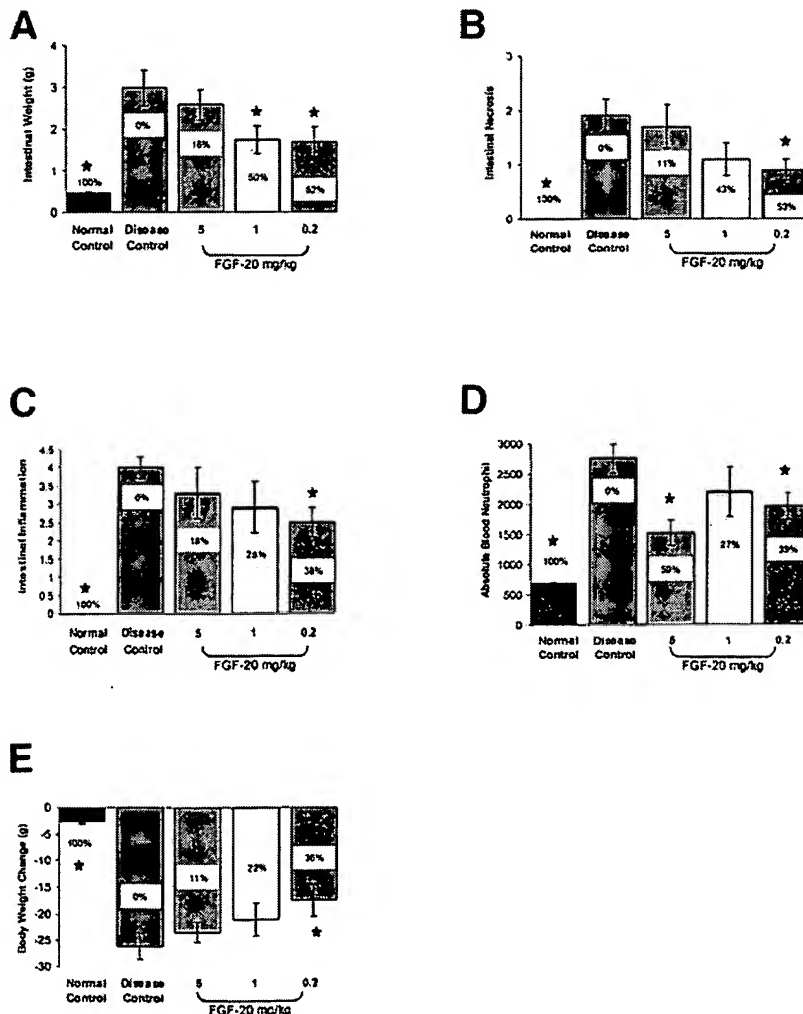


Figure 6. Effects of FGF-20 on indomethacin-induced intestinal ulcerations. To induce intestinal lesions, female Lewis rats were given indomethacin (7.5 mg/kg) SC for 2 days (day 0 to 1). Disease control animals ($n = 8$) received daily IV injections of vehicle solution on day -1 to day 4. FGF-20-treated groups ($n = 8$ animals/group) received daily IV injections of FGF-20 at the indicated concentrations on day -1 to day 4. Normal control animals ($n = 4$) did not receive indomethacin, but did receive daily IV injections of vehicle solution on day -1 to 4. Animals were sacrificed on day 5, and a 10 cm section of the distal jejunum in the area at risk for lesions was (A) weighed and histologically examined and scored for level of (B) necrosis and (C) inflammation. (D) The absolute neutrophil counts obtained from blood harvested at necropsy. (E) The change in total body weight from day 0 to 5. Results are reported as mean \pm standard error, and the percentage changes indicated are relative to the disease control group. Changes that are statistically different from the disease control group ($P < 0.05$ by the 2-tailed Student t test) are indicated with a star.

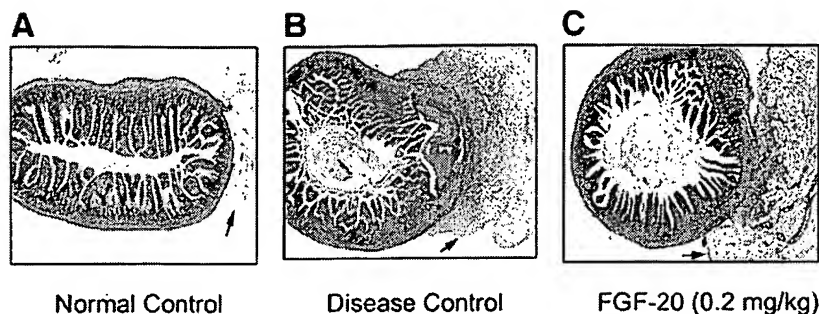


Figure 7. Effects of FGF-20 on indomethacin-induced intestinal ulcerations: histopathology. Representative sections of the distal jejunum from the area at risk were collected at necropsy, preserved in formalin, stained with H&E, magnified 25 \times , and photographed. The groups are as described in the legend to Figure 6. Arrows indicate attached mesentery. Note that FGF-20 (0.2 mg/kg) inhibits the necrosis associated with indomethacin treatment.

ng/mL) stimulated PGE₂ production to a similar degree as that of the positive control (10% FBS).

Discussion

FGF-20 is a novel growth factor that exhibits proliferative activity on fibroblasts and epithelial cells.¹⁹ Because both of these cell types play important roles in

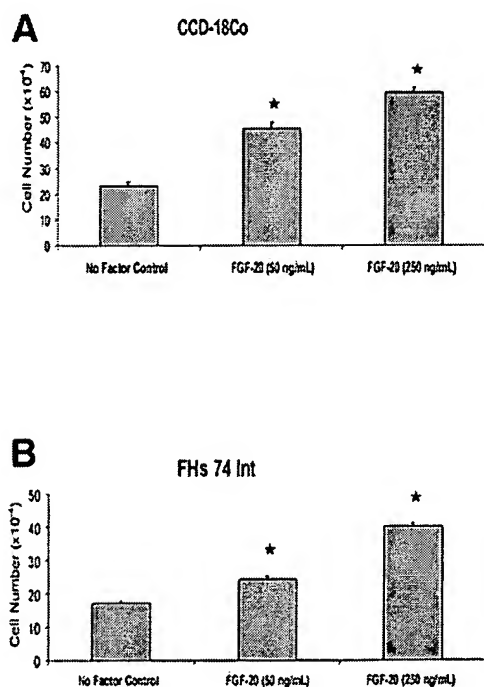


Figure 8. Effects of FGF-20 on the growth of human intestinal cells in vitro. CCD-18Co human colonic fibroblasts (A) and FHs 74 Int human intestinal epithelial cells (B) were cultured without or with the indicated concentrations of FGF-20 for 6 days and then counted. Data points represent the mean of duplicate wells \pm standard deviation. Changes that are statistically different from control cells receiving no factor ($P < 0.05$ by the 2-tailed Student *t* test) are indicated with a star. The experiment was performed 2 times with similar results.

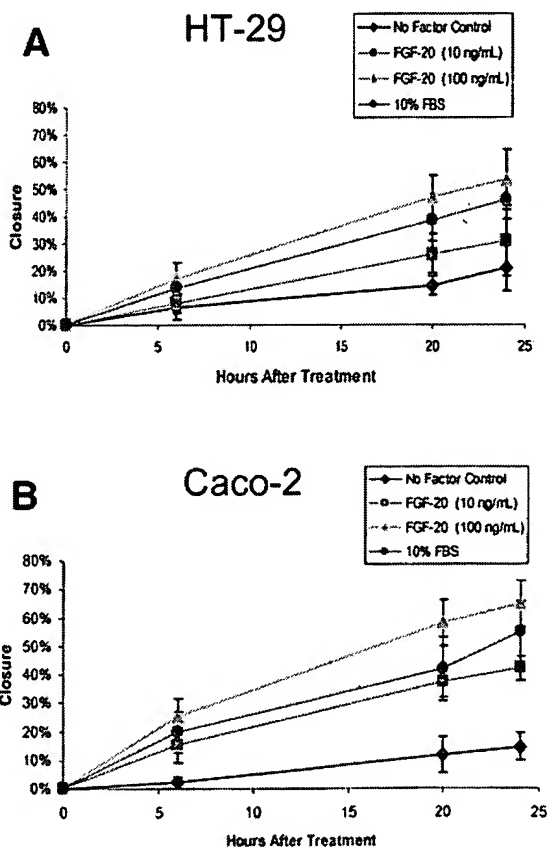


Figure 9. Effects of FGF-20 on the restitution of human colonic cells in vitro. Monolayers of "wounded" HT-29 (A) and Caco-2 (B) human colonic epithelial cancer cells were cultured without or with the indicated concentrations of FGF-20, or 10% FBS as a positive control, and wound width was measured after 0, 6, 20, and 24 hours. Each data point represents the mean of 12 wounds \pm standard deviation, and results are reported as the percentage of closure relative to values obtained at time 0. All FGF-20 data points, with the exception of the treatment of HT-29 with 10 ng/mL FGF-20 for 6 hours, are statistically different ($P < 0.05$ by the 2-tailed Student *t* test) from control cells receiving no factor. The experiment was performed 3 times with similar results.

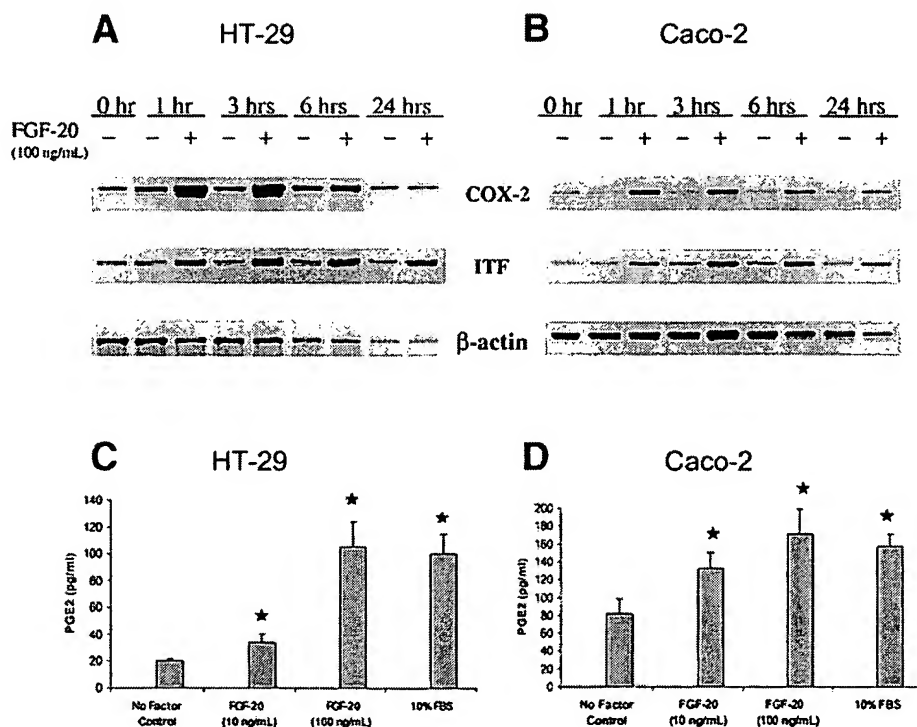


Figure 10. Effects of FGF-20 on the expression of COX-2 and ITF mRNA and on PGE₂ levels in human colonic cells in vitro. The expression of COX-2, ITF, and β -actin RNA transcripts in HT-29 (A) and Caco-2 (B) human epithelial cancer cells cultured without or with FGF-20 (100 ng/mL) for the indicated time periods was determined by RT-PCR. PGE₂ levels in the media of HT-29 (C) and Caco-2 (D) cells cultured for 24 hours without or with the indicated concentrations of FGF-20, or 10% FBS as a positive control, were determined by ELISA. Each data point represents the mean of 9 wells \pm standard deviation. Changes that are statistically different from control cells receiving no factor ($P < 0.05$ by the 2-tailed Student *t* test) are indicated with a star. The experiment was performed 3 times with similar results.

tissue repair,⁴ we sought to examine the effect of FGF-20 in a tissue repair model. To this end, we initiated a series of experiments to explore the effect of FGF-20 in animal models of IBD, a disease in which the integrity of the intestinal epithelium is compromised¹⁻³ and for which additional therapies are needed. Further rationale for assessing the activity of FGF-20 in IBD animal models originates from the finding that FGF-20 interacts strongly with various FGFRs, including FGFR2b, FGFR2c, and FGFR3c,¹⁹ at least 1 of which is present on intestinal epithelium,^{7,8} and from previous studies implicating FGFs in intestinal epithelial repair.^{7,13-18,25}

The present findings demonstrate that FGF-20 is active in 2 independent rodent IBD models: DSS treatment of mice to induce an ulcerative colitis-like syndrome, and indomethacin treatment of rats to induce a Crohn's-like disease consisting of inflammation and ulceration of the small bowel. The decision to use these 2 models was based on the fact that each model affects a different region of the gastrointestinal tract and thus may represent different human disease counterparts. Moreover, the pathologies associated with these models are highly reproducible.²²

In the DSS model, a significant beneficial effect of FGF-20, as indicated by various experimental parameters, was evident when this growth factor was administered concomitantly with DSS. This effect was seen regardless of whether FGF-20 was administered through the IP or the SC route. In addition, a beneficial effect of FGF-20 on animal survival was found when FGF-20 was administered therapeutically after 4 days of DSS exposure. The protective effects of FGF-20 in the DSS model occurred in a dose-dependent fashion, with maximum protection observed at the highest concentration examined (5 mg/kg). FGF-20-administered IV also proved active in the rat indomethacin model, in which an inverse dose-response was observed. Bimodal dose-response curves have been reported for other biological molecules,^{13,26,27} and it is possible that the activity of FGF-20 in the DSS and indomethacin models fall on opposite sides of the response curve.

The in vitro studies presented herein suggest multiple mechanisms for explaining how FGF-20 stimulates intestinal healing. For example, FGF-20 may be enhancing mucosal repair, a hypothesis supported by

the finding that this factor increases the restitution of colonic epithelial cells and the growth of colonic fibroblasts and intestinal epithelial cells. Because ITF has been shown to accelerate epithelial restitution^{24,28} and FGF-20 increases ITF expression in colonic epithelia, it is also possible that ITF mediates at least some of the mucosal repair induced by FGF-20. Additional properties of ITF that may contribute to the support of the mucosal barrier include its ability to increase mucus viscosity²⁹ and prevent epithelial cell apoptosis.³⁰ The data further suggest that FGF-20 may enhance mucosal healing by stimulating a COX-2-mediated increase in PGE₂, a molecule which has been shown to stimulate the healing process.^{23,31} The net effect of accelerated epithelial restitution, restoration of an intact epithelial layer, and improved mucosal barrier function after FGF-20 treatment is decreased mucosal permeability and reduced inflammation caused by decreased uptake of inflammation-inducing substances, including bacterial antigens, cell wall polymers, and chemotactic peptides.¹ Moreover, PGE₂ (generated by COX-2 by way of FGF-20) may reduce inflammation by inhibiting inflammatory cell activation.³²

There are currently no marketed drugs for IBD that stimulate intestinal repair, although at least 2 FGF family members (FGF-7 and FGF-10, also known as KGF-1 and KGF-2, respectively), have shown activity in animal models of IBD.^{13,17} Because of their narrow receptor specificity, FGF-7 and FGF-10 efficiently activate some epithelial cells, but not fibroblasts.³³ However, FGF-20 interacts with a variety of FGF receptors¹⁹ and thus is capable of activating fibroblasts as well as epithelial cells. Evidence suggests that fibroblasts play an important role in the repair of epithelium,⁴ and thus FGF-20 may be well suited for the treatment of this disease.

A biological agent for the treatment of IBD, an antibody to TNF- α , has proven useful in the treatment of moderate to severe Crohn's disease.³⁴ However, use of this product increases the risk of infection.³⁴ In contrast, FGF-20 represents a new class of biological agent that may prove effective in the treatment of IBD by stimulating intestinal repair without increasing the risk of infection.

References

1. Sartor RB. Microbial factors in the pathogenesis of Crohn's disease, ulcerative colitis and experimental intestinal inflammation. Philadelphia: Saunders, 1999.
2. Hyams JS. Inflammatory bowel disease. *Pediatr Rev* 2000;21:291-295.
3. Fiocchi C. Inflammatory bowel disease: etiology and pathogenesis. *Gastroenterology* 1998;115:182-205.
4. Dignass AU. Mechanisms and modulation of intestinal epithelial repair. *Inflamm Bowel Dis* 2001;7:68-77.
5. Beck PL, Podolsky DK. Growth factors in inflammatory bowel disease. *Inflamm Bowel Dis* 1999;5:44-60.
6. Szebenyi G, Fallon JF. Fibroblast growth factors as multifunctional signaling factors. *Int Rev Cytol* 1999;185:45-106.
7. Housley RM, Morris CF, Boyle W, Ring B, Biltz R, Tarpley JE, Aukerman SL, Devine PL, Whitehead RH, Pierce GF. Keratinocyte growth factor induces proliferation of hepatocytes and epithelial cells throughout the rat gastrointestinal tract. *J Clin Invest* 1994;94:1764-1777.
8. Hughes SE. Differential expression of the fibroblast growth factor receptor (FGFR) multigene family in normal human adult tissues. *J Histochem Cytochem* 1997;45:1005-1019.
9. Brauchle M, Madlener M, Wagner AD, Angermeyer K, Lauer U, Hofschneider PH, Gregor M, Werner S. Keratinocyte growth factor is highly overexpressed in inflammatory bowel disease. *Am J Pathol* 1996;149:521-529.
10. Thorn M, Raab Y, Larsson A, Gerdin B, Hallgren R. Intestinal mucosal secretion of basic fibroblast growth factor in patients with ulcerative colitis. *Scand J Gastroenterol* 2000;35:408-412.
11. Bajaj-Elliott M, Breese E, Poulsom R, Fairclough PD, MacDonald TT. Keratinocyte growth factor in inflammatory bowel disease. Increased mRNA transcripts in ulcerative colitis compared with Crohn's disease in biopsies and isolated mucosal myofibroblasts. *Am J Pathol* 1997;151:1469-1476.
12. Finch PW, Pricolo V, Wu A, Finkelstein SD. Increased expression of keratinocyte growth factor messenger RNA associated with inflammatory bowel disease. *Gastroenterology* 1996;110:441-451.
13. Han DS, Li F, Holt L, Connolly K, Hubert M, Miceli R, Okoye Z, Santiago G, Windle K, Wong E, Sartor RB. Keratinocyte growth factor-2 (FGF-10) promotes healing of experimental small intestinal ulceration in rats. *Am J Physiol Gastrointest Liver Physiol* 2000;279:G1011-G1022.
14. Potten CS, O'Shea JA, Farrell CL, Rex K, Booth C. The effects of repeated doses of keratinocyte growth factor on cell proliferation in the cellular hierarchy of the crypts of the murine small intestine. *Cell Growth Differ* 2001;12:265-275.
15. Dignass AU, Tsunekawa S, Podolsky DK. Fibroblast growth factors modulate intestinal epithelial cell growth and migration. *Gastroenterology* 1994;106:1254-1262.
16. Egger B, Procaccino F, Sarosi I, Tolmos J, Buchler MW, Eysselein VE. Keratinocyte growth factor ameliorates dextran sodium sulfate colitis in mice. *Dig Dis Sci* 1999;44:836-844.
17. Miceli R, Hubert M, Santiago G, Yao DL, Coleman TA, Huddleston KA, Connolly K. Efficacy of keratinocyte growth factor-2 in dextran sulfate sodium-induced murine colitis. *J Pharmacol Exp Ther* 1999;290:464-471.
18. Zeeh JM, Procaccino F, Hoffmann P, Aukerman SL, McRoberts JA, Soltani S, Pierce GF, Lakshmanan J, Lacey D, Eysselein VE. Keratinocyte growth factor ameliorates mucosal injury in an experimental model of colitis in rats. *Gastroenterology* 1996;110:1077-1083.
19. Jeffers M, Shinkets R, Prayaga S, Boldog F, Yang M, Burgess C, Fernandes E, Rittman B, Shinkets J, LaRochelle WJ, Lichenstein HS. Identification of a novel human fibroblast growth factor and characterization of its role in oncogenesis. *Cancer Res* 2001;61:3131-3138.
20. Rosenberg IM, Goke M, Kanai M, Reinecker HC, Podolsky DK. Epithelial cell kinase-B61: an autocrine loop modulating intestinal epithelial migration and barrier function. *Am J Physiol* 1997;273:G824-G832.
21. Okayasu I, Hatakeyama S, Yamada M, Ohkusa T, Inagaki Y, Nakaya R. A novel method in the induction of reliable experimen-

- tal acute and chronic ulcerative colitis in mice. *Gastroenterology* 1990;98:694-702.
22. Elson CO, Sartor RB, Tennyson GS, Riddell RH. Experimental models of inflammatory bowel disease. *Gastroenterology* 1995; 109:1344-1367.
 23. Wallace JL. Prostaglandin biology in inflammatory bowel disease. *Gastroenterol Clin North Am* 2001;30:971-980.
 24. Podolsky DK. Mechanisms of regulatory peptide action in the gastrointestinal tract: trefoil peptides. *J Gastroenterol* 2000; 35(Suppl 12):69-74.
 25. Iwakiri D, Podolsky DK. Keratinocyte growth factor promotes goblet cell differentiation through regulation of goblet cell silencer inhibitor. *Gastroenterology* 2001;120:1372-1380.
 26. Targan SR, Hanauer SB, van Deventer SJ, Mayer L, Present DH, Braakman T, DeWoody KL, Schaible TF, Rutgeerts PJ. A short-term study of chimeric monoclonal antibody cA2 to tumor necrosis factor alpha for Crohn's disease. Crohn's Disease cA2 Study Group. *N Engl J Med* 1997;337:1029-1035.
 27. Schreiber S, Fedorak RN, Nielsen OH, Wild G, Williams CN, Nikolaus S, Jacyna M, Lashner BA, Gangl A, Rutgeerts P, Isaacs K, van Deventer SJ, Koningsberger JC, Cohard M, LeBeaut A, Hanauer SB. Safety and efficacy of recombinant human interleukin 10 in chronic active Crohn's disease. Crohn's Disease IL-10 Cooperative Study Group. *Gastroenterology* 2000;119:1461-1472.
 28. Kato K, Chen MC, Nguyen M, Lehmann FS, Podolsky DK, Soll AH. Effects of growth factors and trefoil peptides on migration and replication in primary oxynitic cultures. *Am J Physiol* 1999;276 (5 Part 1):G1105-G1116.
 29. Kindon H, Pothoulakis C, Thim L, Lynch-Devaney K, Podolsky DK. Trefoil peptide protection of intestinal epithelial barrier function: cooperative interaction with mucin glycoprotein. *Gastroenterology* 1995;109:516-523.
 30. Taupin DR, Kinoshita K, Podolsky DK. Intestinal trefoil factor confers colonic epithelial resistance to apoptosis. *Proc Natl Acad Sci U S A* 2000;97:799-804.
 31. Cohn SM, Schloemann S, Tessner T, Seibert K, Stenson WF. Crypt stem cell survival in the mouse intestinal epithelium is regulated by prostaglandins synthesized through cyclooxygenase-1. *J Clin Invest* 1997;99:1367-1379.
 32. Marcinkiewicz J. In vitro cytokine release by activated murine peritoneal macrophages: role of prostaglandins in the differential regulation of tumor necrosis factor alpha, interleukin 1, and interleukin 6. *Cytokine* 1991;3:327-332.
 33. Igarashi M, Finch PW, Aaronson SA. Characterization of recombinant human fibroblast growth factor (FGF)-10 reveals functional similarities with keratinocyte growth factor (FGF-7). *J Biol Chem* 1998;273:13230-13235.
 34. Nightingale SL. From the Food and Drug Administration. *JAMA* 1998;280:1128.

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